Patent Application
Docket No. USF-P173CXC1
Serial No. 09/914,508

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Vera Afremova
Art Unit	:	1651
Applicant	:	Beerelli Seshi
Serial No.	:	09/914,508
Filed	:	November 7, 2001
For	:	Human Mesenchymal Progenitor Cell

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF BEERELLI SESHI, M.D., UNDER 37 C.F.R. §1.132

Sir:

I, Beerelli Seshi, M.D., hereby declare:

THAT, my *curriculum vitae*, including published works, is attached hereto as Exhibit A;

THAT, I am the inventor on the above-referenced patent application;

THAT, I have read and understood the specification and claims of the subject application and the Office Actions dated April 25, 2003 and October 10, 2003;

AND, being thus duly qualified, do further declare:

The Office Action dated October 10, 2003 indicates that claims 50-62 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. At pages 2 and 3, the Office Action asserts that the above-referenced patent application does not provide support for the concept that "each" cell or "at least 95%" of the cells simultaneously express a plurality of genes that are markers for multiple cell lineages comprising at least four different mesenchymal cell lineages, such as adipocyte, osteoblast, fibroblast, and muscle cell. This is incorrect. As acknowledged at page 3 of the Office Action, 85% of the cells tested expressed the fourth mesenchymal cell lineage marker, the muscle cell marker actin. However, the pluri-differentiated mesenchymal progenitor cells of the invention express other muscle-specific markers,

such as caldesmon and transgelin, as determined by Northern blot analysis and described at page 9, lines 20-21, and page 32, lines 11-15, of the subject specification. As indicated at page 32, lines 24-27, of the specification, the morphologic, cytochemical and immunohistochemical results, and the Northern blot data disclosed in the specification, the cells of the present invention co-express markers specific for at least four different mesenchymal cell lineages. Thus, the cells of the invention express the full spectrum of mesenchymal cell markers recited in the claims, and the cells are homogenous in this regard. Furthermore, as described at pages 15 and 16 of the patent application, isolation of the pluri-differentiated mesenchymal progenitor cells from macrophages and hematopoietic cells of the Dexter culture to a purity of approximately 95% was demonstrated. However, it would be immediately envisioned by those of ordinary skill in the art that the remaining 5% of contaminating cells (macrophages and hematopoietic cells) could be removed using methods known in the art, such as immunomagnetic separation (IMS) techniques, thereby achieving a purity of greater than 99%. Investigators have successfully used immunomagnetic beads to separate and enrich carcinoma cells from bone marrow and peripheral blood for some time (Naume *et al.*, 1997, "Immunomagnetic Techniques for the Enrichment and Detection of Isolated Breast Carcinoma Cells in Bone Marrow and Peripheral Blood", *J. Hematother.*, 6(2):103-114; Naume *et al.*, 1998, "Increased Sensitivity for Detection of Micrometastases in Bone-Marrow/Peripheral-Blood Stem-Cell Products from Breast-Cancer Patients by Negative Immunomagnetic Separation", *Int. J. Cancer*, 78(5):556-560; Shibata, K. *et al.*, 1998, "Detection of Ras Gene Mutations in Peripheral Blood of Carcinoma Patients Using CD45 Immunomagnetic Separation and Nested Mutant Allele Specification Amplification", *Int. J. Oncol.*, 12(6):1333-1338).

The experimental data disclosed in the patent application is also described in the Seshi *et al.* (2000) publication (*Blood Cells, Molecules, and Diseases*, 26(3):234-246), of which I am the first author. My findings concerning the pluri-differentiation exhibited by the mesenchymal progenitor cells of the invention have been acknowledged within the scientific community, as demonstrated by subsequent publications that reference this work, such as Siler *et al.* (see page 219, first column, lines 4) and Woodbury *et al.* (see page 915, second column, 22-34).

The fact that the pluri-differentiated mesenchymal progenitor cells of the invention express four lineage-specific mesenchymal cell markers, including muscle cell markers, was also confirmed in the Seshi *et al.* (2003) publication (*Blood Cells, Molecules, and Diseases*, 31:268-285), of which I am the first author. The Seshi *et al.* (2003) publication describes microarray analysis of the mesenchymal progenitor cells of the invention at the single-cell level. As shown in the gene expression plots of Figures 6A and 6B, and as outlined in Table 2 (A-D) of the Seshi *et al.* (2003) publication, isolated single cells simultaneously expressed genes specific for four different mesenchymal cell lineages (osteoblast, muscle, fibroblast, and adipocyte). Figure 6A of the publication shows that all ten single-cell samples expressed both muscle cell markers caldesmon and transgelin. These results support the concept that each of the mesenchymal progenitor cells of the invention express the full repertoire of mesenchymal cell markers recited in the claims (*i.e.*, at least four different mesenchymal cell lineages, wherein each of the markers is specific for a single cell lineage). Because this feature can be reasonably attributed to each of the cells, they are homogenous in this regard.

The Office Action dated October 10, 2003 indicates that claims 42-62 are rejected under 35 U.S.C. §102(b) as anticipated by the Torok-Storb *et al.* patent (U.S. Patent No. 5,879,940). The invention of the subject patent application is based upon the discovery that there are only three types of cells in Dexter-type cultures (also known as long-term marrow cultures (LTMC)): macrophages, hematopoietic cells, and the non-hematopoietic cells of the present invention, which simultaneously express marker genes specific for multiple mesenchymal cell lineages, including adipocytes, osteoblasts, fibroblasts, and smooth muscle cells. Prior to this, the prevailing belief was that stromal cells of Dexter-type cultures were a heterogenous mixture of adipocytes, osteoblasts, fibroblasts, muscle cells, and vascular endothelial cells. Thus, the cells of the present invention are obtainable from primary Dexter-type cell cultures and simultaneously express a plurality of genes that are markers for multiple cell lineages without the need for genetic modification.

In contrast, the Torok-Storb *et al.* patent describes several bone marrow stromal cell lines that have been transformed with human papilloma virus (HPV). Although the cells of the Torok-Storb *et al.* patent and the cells of the present invention both originate from Dexter-type cultures (as prepared in Gartner and Kaplan, *Blood* 56:117, 1980), that is where their similarity ends. As described at column 11, lines 38-65, of the Torok-Storb *et al.* patent, LTMC (Dexter-type cultures) were established. The LTMC are a heterogeneous mixture of macrophages, hematopoietic cells, and non-hematopoietic cells, as taught in the subject specification. The primary LTMC were then immortalized with HPV and transduced clones were selected and expanded, resulting in the immortalized stromal cell lines of the Torok-Storb *et al.* patent (column 11, lines 60-62). In contrast, the pluri-differentiated cells of the present invention were isolated from primary cultures (Dexter-type cultures) by removal of hematopoietic cells and macrophages (as described at page 16, lines 11-33, of the subject specification), and subsequently characterized. No immortalization or other genetic modification was undertaken.

The immortalized stromal cell lines of the Torok-Storb *et al.* patent were qualitatively analyzed by immunocytochemistry, and the results do not indicate that any of the cells of the immortalized cell lines simultaneously expresses genes that are markers of at least four different mesenchymal cell lineages, wherein each of the markers is specific for a single cell lineage, as recited in the claims. For sake of clarity, I have summarized the qualitative results reported by Torok-Storb *et al.* pertaining to the mesenchymal cell markers in Table A below.

Table A.

	Immortalized Stromal Cell Lines of Torok-Storb *et al.*			
Mesenchymal cell marker (lineage specificity)	HS-5	HS-21	HS-23	HS-27
Actin (muscle)	+++	+++	+++	+++
Fibronectin (fibroblast)	++	++	++	++
Alkaline Phosphatase (osteoblast)	-	+/-	+/-	+/-

Oil Red O (adipocyte)	- (Did not accumulate lipids)	- (Did not accumulate lipids)	Lipid vacuoles in presence of dexamethasone only; but not the large multilocular vacuoles observed in LTMCs.	A few cells (approx. 1-2%) formed lipid vacuoles in presence of steroids.

As shown in Table A, and described at columns 13 and 14 of the Torok-Storb *et al.* patent, four cell lines (HS-5, HS-21, HS-23, and HS-27) were qualitatively tested for the expression of a variety of markers. Only four of the markers tested are mesenchymal markers that are specific for a single cell lineage, as recited in the claims of the subject application. These are the muscle marker actin, the fibroblast marker fibronectin, the osteoblast marker alkaline phosphatase, and the adipocyte marker Oil Red O. It should be understood that the markers vimentin, Collagen I, Collagen II, and Collagen III, for example, are each specific for a variety of mesenchymal cell lineages, such as lymphoid cells, fibroblasts, endothelial cells, and smooth muscle cells.

Interpreting the qualitative data using the legend in Table 1 of the Torok-Storb *et al.* patent, all four cell lines were "strongly positive" for the muscle marker actin, and showed "good staining" for the fibroblast marker fibronectin. However, the cells of the HS-5 cell line did not express the markers specific for osteoblasts (Table 1) or adipocytes (column 14, lines 43-44). Furthermore, the cells of the HS-21 cell line were only "heterogeneously positive" for the osteoblast marker (Table 1), and did not express the marker for adipocytes (column 14, lines 43-44). Clearly, the cells of the HS-5 cell line and HS-21 cell line do not simultaneously express markers of least four different mesenchymal cell lineages, wherein each marker is specific for a single cell lineage.

The Torok-Storb *et al.* patent indicates that cells of the HS-23 cell line were only "heterogeneously positive" for the osteoblast marker (Table 1) and formed lipid vacuoles only in the presence of dexamethasone, but not in the presence of other steroids and not to the extent of the

large multilocular vacuoles observed in LTMCs (see column 14, lines 46-51). Likewise, the cells of the HS-27 cell line were only "heterogeneously positive" for the osteoblast marker and "only a few cells (approximately 1-2%)" of the cell line formed lipid vacuoles (see column 14, lines 44-45). The qualitative data reported in the Torok-Storb *et al.* patent do not indicate that any cells within the HS-23 or HS-27 cell lines simultaneously express all four markers, as recited in the claims of the subject application. The fact that the cells of the HS-23 and HS-27 cell lines only had "good staining" of the fibroblast marker, were only "heterogeneously positive" for the osteoblast marker, and had very limited expression of the adipocyte marker does not convey to one of ordinary skill in the art that any cells within the cell lines simultaneously expresses these markers. For example, in order for any cells of the HS-27 cell line to simultaneously express all four markers, it would have to be shown that the "few cells (approximately 1-2%)" that formed lipid vacuoles were also among those positive for the osteoblast marker. Because the cells were only "heterogeneously positive" for the osteoblast marker, cells that were positive for the adipocyte marker and osteoblast marker may be mutually exclusive. Therefore, simultaneous expression of all four markers cannot be attributed to the cells.

In contrast, as shown in Table 1 of the subject application, it has been demonstrated that all of the mesenchymal progenitor cells expressed the adipocyte marker (Nile Red), all of the cells expressed the osteoblast marker (alkaline phosphatase), all of the cells expressed the fibroblast marker (fibronectin and prolyl-4-hydroxylase), and 85% of the cells expressed the muscle marker (actin). Therefore, necessarily, those cells expressing the muscle marker must have also expressed the other three mesenchymal cell markers. This is not the case for the Torok-Storb *et al.* cell lines. Furthermore, as indicated above, the Seshi *et al.* (2003) publication contains experimental data verifying the fact that the cells of the present invention individually express at least four different mesenchymal-specific cell markers, including an adipocyte marker.

Presumably, the Examiner is relying on the premise that because the cells in the Torok-Storb *et al.* patent are cell lines, the cells within each cell line are homogenous and share the same phenotype. However, the intra-cell line heterogeneity in the expression of the markers in Table 1 is

inconsistent with this premise. For example, the significant heterogeneity in the expression of the osteoblast and adipocyte markers may suggest non-specific staining (a false-positive), or may indicate that the cell lines are unstable or have lost their monoclonality. This latter possibility is made more likely by Example 1 of the Torok-Storb *et al.* patent itself. Column 12, lines 53-57, of the Torok-Storb *et al.* patent indicates that Southern hybridization produced autoradiographs with two bands for each of the HS-21 and HS-27 cell lines, "indicating either that they contained two inserts or that two clones contribute to the line" (emphasis added).

The Graf *et al.* publication (*Blood*, 100(4):1509-1511, 2002) describes gene expression profiling of the HS-5 cell line and HS-27a cell line, a sub-clone of the HS-27 cell line, using DNA microarray technology. There is no data within the Graf *et al.* publication indicating that the cells of the HS-27a subclone simultaneously express the four mesenchymal-specific markers tested for in the Torok-Storb *et al.* patent. Thus, the Graf *et al.* publication lends no support to the homogeneity of the parent cell line (HS-27).

Therefore, based on the heterogeneity in the markers expressed by the cells within each immortalized cell line of the Torok-Storb *et al.* patent, one would not reasonably conclude that any one cell simultaneously expresses genes that are markers of at least four different mesenchymal cell lineages, wherein each of the markers is specific for a single cell lineage.

To the extent that the Office Action asserts that the immortalized cells of the Torok-Storb *et al.* patent are characterized by "the same phenotype" as pluri-differentiated cells of the present invention, it should be understood that the scientific literature indicates that the phenotypic characteristics of these immortalized cells are unpredictable. For example, citing Roecklein and Torok-Storb (*Blood*, 85:997-1005, 1995), the Majumdar *et al.* publication, which is relied upon by the Examiner in the instant Office Action, indicates that unpredictable changes in phenotype may occur in immortalized bone marrow stromal cells, such as those immortalized with human papilloma virus E6/E7 genes, which is the same retroviral construct used in the Torok-Storb *et al.* patent (see

page 57, paragraph bridging columns 1 and 2 of Majumdar *et al.*; and column 11, lines 56-59, of the Torok-Stork *et al.* patent). Specifically, Majumdar *et al.* teaches that while immortalized bone marrow stromal cells have been used in long-term bone marrow cultures to further define the heterogeneity in the marrow microenvironment,

> the major disadvantage of relying on transformed and immortalized cell lines to determine the functional elements of the marrow microenvironment lies in the potential of these cells to undergo morphologic, phenotypic, and regulatory changes that make them unpredictable surrogates for their normal cell counterparts (emphasis added).

The Roecklein and Torok-Storb *et al.* publication (*Blood*, 85(4):997-1005, 1995) describes the work on which the Torok-Storb *et al.* patent is based. As indicated at page 1001, column 2, of the Torok-Storb *et al.* (1995) publication, epithelial cells immortalized by the HPV-16 E6 and E7 genes exhibit aneuploidy (having an abnormal chromosome complement) in late passage cells. The ability of HPV-16 E6 and E7 proteins to induce numerical and structural chromosome instability has been acknowledged elsewhere in the literature, such as Deunsing *et al.* (PNAS, 97(18):10002-10007, 2000) and Deunsing and Munger (Cancer Research, 62:7075-7082, 2002).

Furthermore, the Torok-Storb *et al.* patent does not teach or suggest using the immortalized stromal cell lines as therapeutic agents within a pharmaceutical composition. The Torok-Storb *et al.* patent proposes that the immortalized stromal cell lines be used to sustain and expand hematopoietic precursor cells *in vitro*, where the hematopoietic precursor cells are harvested and subsequently returned to a patient, or frozen and stored (see abstract, column 3, lines 39-42). Alternatively, the immortalized cell lines can be used as feeder layers in *ex vivo* bone marrow cultures or in colony forming assays, or medium conditioned by exposure to the immortalized cell lines may be used *in vivo* to promote hematopoiesis (see abstract, column 3, lines 58-61, and column 4, lines 42-53). The Torok-Storb *et al.* patent does not teach or suggest using the immortalized cell lines in a pharmaceutical composition or otherwise administering the immortalized cell lines to a patient.

The Office Action refers to a co-culture containing hematopoietic cells and the immortalized stromal cell lines, as described at column 15, lines 63-67, of the Torok-Storb *et al.* patent. While "serum-deprived medium" may be considered a "carrier" in its broadest sense, it is not necessarily a "pharmaceutically acceptable" carrier, absent a description of its ingredients. Furthermore, the mere fact that the co-culture was observed to support the growth of hematopoietic cells *in vitro* does not necessarily correlate with an amount of cells effective for treating a disease state *in vivo*, for enhancing hematopoietic stem cell engraftment, or for treating graft-versus-host disease, as recited in the claims of the subject application. Therefore, the Torok-Storb *et al.* patent does not teach the pluri-differentiated cells or pharmaceutical compositions of the present invention.

The Office Action dated October 10, 2003 indicates that claims 42-62 are rejected under 35 U.S.C. §103(a) as being unpatentable over Majumdar *et al.* taken with Torok-Storb *et al.* (U.S. Patent No. 5,879,940) and Bordignon *et al.* At page 7, the Office Action states that the mesenchymal progenitor cells of Majumdar *et al.* are of the "same cell population" as Torok-Storb *et al.* and are, therefore, capable of simultaneously expressing markers of four different mesenchymal cell lineages including adipocyte, osteoblast, fibroblast, and muscle cells. However, as indicated above, based upon the heterogeneity in the markers expressed by the immortalized cells within each cell line of Torok-Storb *et al.*, one skilled in the art would not conclude that any of the cells simultaneously expresses genes that are markers of at least four different mesenchymal cell lineages, wherein each of the markers is specific for a single cell lineage. Moreover, Majumdar *et al.* teaches that the immortalized cells of the Torok-Storb *et al.* patent are "unpredictable surrogates" for their normal cell counterparts (see page 57, paragraph bridging columns 1 and 2 of Majumdar *et al*). Thus, one of ordinary skill in the art would not consider the immortalized cells of the Torok-Storb *et al.* patent to be of the "same cell population" as Majumdar *et al.*, despite their common origin within bone marrow. Again, prior to my discovery to the contrary, the prevailing belief was that stromal cells of Dexter-type cultures were a heterogenous mixture of adipocytes, osteoblasts, fibroblasts, muscle cells, and vascular endothelial cells. As a result of this perceived cellular complexity, research efforts in the preceding years were not directed to characterizing or isolating the pluri-

differentiated mesenchymal progenitor cells from the Dexter-type cultures. As indicated at page 14, lines 7-23, of the patent application, almost all of the published studies on Dexter-type cultures involved cytochemical and immunocytochemical staining on layers of stromal cells grown to confluence on coverslips. In this "cobblestone" arrangement, the cells appear very complex, with the macrophages and non-hematopoietic cells spreading and assuming various shapes, preventing a clear morphological visualization or characterization of the cells for any particular marker. Only rarely have investigators prepared a cell suspension of Dexter-type cultures and stained the cells on cytospins, as described in Simmons *et al.* (*Nature*, 1987, 328:429-432). Although these methods allow visualization of individual cells, the resulting cytospin preparations still contain the cell types that constitute Dexter-type cultures (*i.e.*, macrophages, hematopoietic cells, and pluri-differentiated mesenchymal progenitor cells). At the time, Simmons *et al.* observed a predominance of two distinct cell populations in the cytospin preparations: macrophages and more heterogeneous appearing cells which they deemed "stromal cells", as discussed at page 429 (column 2) and Figure 1 of Simmons *et al.* Thus, the pluri-differentiated mesenchymal progenitor cells of the present invention were not previously isolated or characterized.

The Majumdar *et al.* publication does not teach or suggest isolation of pluri-differentiated cells of the present invention from the MDSC cultures, or their use in pharmaceutical compositions. Likewise, the Bordignon *et al.* publication does not teach or suggest the pluri-differentiated mesenchymal progenitor cells or pharmaceutical compositions of the invention. In fact, it is unlikely that the immortalized cell lines of the Torok-Storb *et al.* patent would be considered candidates for inclusion within a pharmaceutical composition, given the nature of the immortalizing agent utilized. As indicated above, HPV-16 is a high-risk cancer type and expression of the E6 and E7 HPV oncogenes has been strongly associated with cervical carcinoma. Therefore, the Torok-Storb *et al.* patent, the Majumdar *et al.* publication, and the Bordignon *et al.* publication, when taken individually or together, do not teach or suggest the pluri-differentiated mesenchymal progenitor cells and compositions of the invention.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: _____
Beerelli Seshi, M.D.

Date: 03/10/2004